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(71) Applicant (for all designated States except US): UNIVERSITE DU QUEBEC A MONTREAL [CA/CA]; Pavillon Athanas David, D-3502, 1430 St.-Denis, Montreal, Quebec H2X 3J8 (CA).			
(72) Inventors; and			
(75) Inventors/Applicants (for US only): ALLARD, France [CA/CA]; 7257 Beauchesne, St.-Leonard, Quebec H1S 2C7 (CA). SARHAN, Fathey [CA/CA]; 3277 Achim, St.-Laurent, Quebec H4K 1V5 (CA). HOUDÉ, Mario [CA/CA]; 430 Richard, Longueuil, Quebec J4L 2A4 (CA).			
(74) Agents: DUBUC, Jean, H. et al.; Goudreau Gage Dubuc & Martineau Walker, The Stock Exchange Tower, Suite 3400, 800 Place Victoria, P.O. Box 242, Montreal, Quebec H4Z 1E9 (CA).			
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(54) Title: METHOD FOR IMPROVING THE FREEZING TOLERANCE OF PLANTS

(57) Abstract

The accumulation of the osmolyte glycine–betaine is associated with increased freezing tolerance (FT) in wheat. An exogenous application of betaine at room temperature increased their FT by more than 5 °C. The treated plants showed some of the characteristics of cold-acclimated plants and expressed the low temperature responsive gene *Wcor410* encoding a major membrane associated protein. The combined exposure to low temperature and betaine resulted in a cumulative effect on the improvement of FT surpassing the genetic potential of the plant to withstand freezing. This important finding indicates that an exogenous application of betaine before a predicted frost may be exploited to substantially improve cold or freezing tolerance in sensitive plants. Also disclosed is a method of reducing the growth rate of a plant by at least 30 %, which comprises the step of treating the plant with an effective dosage regimen of betaine or derivative thereof which is not lethal, preferably non-toxic to the plant. When growing the spring wheat variety Glenlea, in the presence of 500 mM of glycine betaine for four days, the growth rate thereof was reduced by about 75 %. Another aspect of the present invention is a method of inhibiting the growth of a plant, which comprises the step of treating said plant with a high dose regimen or betaine or derivative thereof. At lethal doses, this method results in killing undesirable plants. Another aspect of the present invention is a method of improving the germination rate of plant seeds at a temperature which is higher than about 0 °C but not lower than the coldest temperature that the plant seeds can withstand, which comprises the steps of administering to the seeds an effective dosage regimen of betaine or derivative thereof, and allowing the same to germinate at cold temperatures.

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Title of the invention**METHOD FOR IMPROVING THE FREEZING TOLERANCE OF PLANTS****Field of the invention**

5 The invention relates to a method to increase the cold or freezing tolerance of plants by cold acclimating the plants and/or by treating the same with betaines.

This invention also relates to the inhibition of the growth or the reduction of the growth rate of plants by treating them with betaines.

10 This invention further relates to the improvement of the germination rate of plant seeds at cold temperatures by treating the same with betaines.

Background of the Invention

Betaine is a non-toxic osmolyte that is thought to play a role in the protection against environmental stresses in particular salinity and drought stress (1, 2). This compound is mostly synthesized in the chloroplast by the enzymes choline monooxygenase and betaine aldehyde dehydrogenase (1). It may accumulate in different cellular compartments to adjust the osmotic balance (3) and increase the stability of protein tertiary structure thus protecting proteins from denaturation (4). *In vitro* studies have shown that betaine can protect membranes of *Beta vulgaris* roots against heat denaturation (5). Several higher plant enzymes were also shown to be protected by betaine from denaturation caused by heat (6) NaCl or KCl (7). Betaine can also stabilize the photosynthetic activity of isolated chloroplasts over time (8) and protect photosystem II against the inhibitory effect of NaCl (9). Interestingly, it was shown that an exogenous application of 25 mM betaine on barley leaves improves recovery after an osmotic stress imposed by polyethylene glycol (-10 bar) (10).

25 Because betaines have been shown to provide some protection to plants from stressful environmental conditions they have been used to treat soils, plants and seeds.

30 WO 95/35022 discloses a method for treating seeds with betaine to enhance seedling growth and protect seeds against adverse environmental conditions. The seeds may be soaked and dried or coated with betaine. The adverse conditions enumerated are water stress, excess NaCl, extreme temperature or pH and heavy metal toxicity. What is not taught are the temperature extremes and the benefits with respect to the rate of germination at low temperatures.

35 WO 96/07320 discloses the application of betaine to improve the yield of grapevines the temperature extremes are between 3°C to 30°C.

In WO 96/41530, different compositions of betaine are disclosed for use in protecting wheat, potato and grapevines against adverse conditions including temperatures between 3°C and 30°C.

These references are silent with regards to freezing temperatures and cold acclimation.

In France Allard's thesis, it is taught that in the wheat cultivar Fredrick, cold acclimation for three days 6 °C/2 °C (day/night) combined with the addition of 1000 mM betaine resulted in the improvement in the freezing tolerance of the plants as tested by measuring the survival rate at -10 °C. The survival rate was shown to be 83%, whereas, when treated with only 1000mM betaine, the survival rate was 51%, when compared to controls. This reference does not teach on the optimal conditions for increasing freezing tolerance. The high amount of betaine used (1000mM) was shown to have a toxic effect to the plant since it was found that it produces chlorosis of the leaves. In addition the cultivar Fredrick having a LT₅₀ (lethal temperature where 50% of the plants die) at -17 °C, testing the plants at -10 °C does not teach how more freezing tolerant is the plant when treated with the combined treatment. It further does not teach the effect on cultivars that genotypically exhibit less freezing tolerance. The decrease in plant growth rate is also described and it is shown to be directly proportional to the amount of betaine administered. When the maximal amount of 1000mM was applied to the cultivar Fredrick, a 29% decreased growth rate occurred when compared to control plants. Finally, in this document there were also teachings relating to the protein WCOR 410. It was taught that the protein WCOR 410 accumulates when plants are subjected to cold-acclimation or by increasing amounts of exogenous betaine. This reference however does not appear to teach a period of cold-acclimation which is sufficient for optimally inducing the expression of the *Wcor 410* gene and improving freezing tolerance.

In the patent publication CA 2,104,142, the present inventors disclose the isolation and sequence of three genes responsive to cold temperature. One of these genes is *Wcor410*. However what is not taught is that the protein WCOR410 is induced by betaine in a manner proportional to the amount of betaine applied and that this protein is involved in promoting freezing tolerance in some plants. It does not teach the benefits of combining cold acclimation and betaine administration.

Statement of the invention

There is now provided a method of increasing cold or freezing tolerance in a plant, which comprises the steps of:

- acclimating said plant to a temperature higher than about 0°C but not lower than the coldest temperature that said plant is capable to withstand, for a time sufficient to induce an optimal cold or freezing tolerance, in said plant, and
- administering betaine or a derivative thereof such as glycine betaine to said plant, in a dosage regimen sufficient to induce the same or different optimal cold or freezing tolerance in said plant;

whereby combined steps of cold-acclimating and administering betaine or derivative thereof increase cold or freezing tolerance of said plant over and above the optimal cold or freezing tolerance induced by each step alone.

Preferably, the dosage regimen does not provide an unacceptable toxicity, more preferably, it is non-toxic to said plant.

Any plant could benefit from such a method, preferably, rosaceae, gramineae and grasses, more preferably, roses, strawberry, golf turf, barley or wheat.

In the two latter plants, the time for cold-acclimating is about four weeks, and the dosage regimen is growing the plants in the presence of a solution of glycine betaine having a concentration lower than about 500 mM, preferably about 250 mM.

In the spring wheat variety Glenlea, in which the optimal freezing tolerance, expressed as the temperature where fifty percent of a plant population die (LT_{50}) is about -8 °C for each step alone, the combined treatment resulted in an increase of freezing tolerance by about 6°C to reach a LT_{50} of about -14°C and further resulted in improving photosynthetic capacity and overall physiology of the plants at cold or freezing temperatures.

The optimal freezing tolerance induced by said each step alone and/or in combination is due at least in part to an increased expression of the gene *Wcor410*.

This invention also relates to the reduction of the growth rate of a plant by at least 30%, which comprises the step of treating the plant with an effective dosage regimen of betaine or derivative thereof which is not lethal, preferably non-toxic to the plant.

When growing the spring wheat variety Glenlea, in the presence of 500 mM of glycine betaine for four days, the growth rate thereof was reduced by about 75%.

Another aspect of the present invention is a method of inhibiting the growth of a plant, which comprises the step of treating said plant with a high dose regimen of betaine or derivative thereof, which may even result in a herbicidal effect.

Another aspect of the present invention is a method of improving the germination rate of plant seeds at a temperature which is higher than about 0°C but

not lower than the coldest temperature that said plant seeds can withstand, which comprises the steps of administering to said seeds an effective dosage regimen of betaine or derivative thereof, and allowing said seeds to germinate at said temperature.

5 Description of the invention

This invention is described hereinbelow by way of specific embodiments and appended figures, which purpose is to illustrate the invention rather than to limit its scope.

Brief description of figures

10 **Figures 1a) and b).** Effect of betaine on FT in the cultivar Glenlea.

Plant survival was determined by the regrowth test as described by Perras and Sarhan (25).

Figure 1a): The survival was evaluated after freezing at -8°C.

15 **Figure 1b):** The LT₅₀ was evaluated after freezing different samples to various temperatures

NA, 12 day-old control non-acclimated plants; 100, 250, and 500, plants treated for 4 days with 100, 250, and 500 mM betaine at 25°C respectively; CA, plants cold-acclimated at 6/2°C for 30 days; CA100 and CA250; plants cold acclimated at 6/2°C for 30 days in the presence of 100 and 250 mM betaine respectively. Standard deviation did not exceed ± 10%.

20 **Figures 2a) and b).** Effect of betaine on LT₅₀ in the cultivar Glenlea

Figure 2a): The freezing test was performed at -8°C.

Figure 2b): The freezing test was performed at -13°C.

25 NA, 12 day-old control non-acclimated plants; 250 plants treated for 4 days with 250 mM betaine. CA, plants cold-acclimated at 6/2°C for 30 days; CA250; plants cold acclimated at 6/2°C for 30 days in the presence of 250 mM betaine.

Figure 3. Accumulation of the WCOR410 protein in response to different betaine concentrations in the spring wheat cultivar Glenlea.

30 Total proteins (5 µg) were separated by SDS-PAGE, transferred to a nitrocellulose membrane and probed with the anti-WCOR410 antibody. NA, 12 day old control non-acclimated plants; 100, 250 and 500, plants treated for 4 days with 100, 250, and 500 mM betaine respectively; CA, Cold acclimated plants.

35 **Figure 4a).** Golf turf was treated three times at a weekly interval in fall with 1 L/m² of 200 mM betaine free base in water. The control area was sprayed with water only. The figure shows the turf regrowth in following early spring for the non-treated control and the betaine-treated area. The upper view shows the treated area. Notice the rapidity

of regrowth and greening in the betaine-treated area. This result reflects a greater and healthier winter survival of betaine-treated turf.

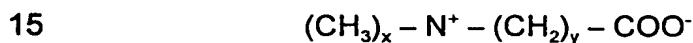
Figure 4b). Close up caption showing the betaine-treated area (upper view) and the non-treated control (bottom).

5 **Figures 5a) and b).** Another example of the effect of betaine on winter survival and regrowth in early spring. The betaine treatment was the same as in Figure 4 except that the application was started earlier in previous fall. Both areas were aerated at the same time. Notice the higher turf density of the betaine-treated area.

Figure 5a). Betaine-treated.

10 **Figure 5b).** Control sprayed with water.

Betaine refers to amino acids where the nitrogen is fully or partly methylated. Betaines are natural products present in plants and animals with a probable function as an osmolyte regulator that protect the cell from osmotic stress. Betaine have the general formula:



where x may be 1 and preferably 2 for cyclic betaine or 3 for straight chain betaines, and y is at least 1. The most common betaine is a glycine derivative where the three methyl groups are attached to the nitrogen of a glycine molecule.

20 Other betaines that are known of which some that are available commercially are presented in Table 1.

TABLE I

	NAMES	OTHER NAMES
	Glycinebetaine	Oxyneurin, betaine
	β -alaninebetaine	Homobetaine
25	2-trimethylamino-6-ketoheptanoate	
	Prolinebetaine	Stachydrene
	Proline	
	N-methyl-L-proline	
	Trans-4-hydroxy-N-methyl-L-proline	
30	Cis-3-hydroxy-N-methyl-L-proline	
	(-)-4-hydroxyproline betaine	Betonicine
	(+)-4-hydroxyprolinebetaine	Turicine
	3-hydroxyprolinebetaine	3-oxystachydrene
	Histidinebetaine	Herzynine, Ercinine
35	Tryptophanbetaine	Hypaphorine

2-mercaptophistidine-betaine	Ergothioneine
Pipecolabetaine	Homostachydine
Nicotinic acid betaine	Trigonelline

Using two wheat cultivars that differ in their levels of freezing tolerance (FT),

5 the role of endogenous betaine was investigated during cold acclimation. In addition, studies on the effect of an exogenous application of betaine on FT alone and in combination with cold acclimation, on the expression of low temperature-responsive genes and on photosynthetic activity have been conducted.

To determine if betaine accumulation is associated with increased FT, the

10 betaine contents were determined in two wheat varieties differing in their FT (cv Glenlea, LT₅₀ (lethal temperature for 50% of the plants) of -8°C and cv Fredrick, LT₅₀ of -17°C). In both cultivars, betaine content decreases during growth at the non-acclimated temperature of 24/20°C while it increases during growth at the cold-acclimating conditions of 6/2°C. The basal betaine level is 30% higher in the more

15 tolerant cultivar Fredrick before cold acclimation (8.5 µmol/g FW in Fredrick compared to 6.5 µmol/g FW in Glenlea). At the end of the acclimation period (where maximal LT₅₀ has been reached) cv Fredrick has accumulated 21.3 µmol/g FW of betaine compared to 15.3 µmol/g FW for cv Glenlea. On a dry weight basis, cv Fredrick has accumulated 106.5 µmol/g DW compared to Glenlea which has accumulated 82.7 µmol/g DW. This

20 result suggests that the increase in betaine content is associated with the development of FT of the two cultivars. A similar increase in betaine was correlated with the FT of different barley cultivars (11). If we calculate the contribution of betaine to the total osmolality of the cell, we find that betaine accounts for only 3.6% and 4.5% of the osmolality after 30 days of cold acclimation for Glenlea and Fredrick respectively. This

25 result demonstrates that betaine contribution to the total osmolality is very low. However, as suggested by Wyn Jones *et al* (12), such a low concentration would require compartmentation in order to play a significant role as osmoprotectant. Studies performed by Matho *et al* (13) have shown that betaine is excluded from vacuoles of spinach leaf cells and is mostly found in the cytoplasm and chloroplasts. It was

30 estimated that betaine concentration can reach 300 mM in spinach (14) and Sueda (8) chloroplasts when plants are submitted to salt stress. This concentration is approximately 20 fold greater than the average betaine leaf concentration. Betaine compartmentation was not determined in wheat but if we consider a similar concentration factor in the chloroplasts during cold acclimation, the actual

35 concentration of betaine could be very significant. Since we have estimated that betaine accounts for 4.5% of the osmolality in cold-acclimated Fredrick, a twenty fold higher concentration of betaine in the chloroplast would mean that betaine contributes for approximately 90% of the chloroplasts' osmolality (or 612 mOsm). Such a

concentration could have a great impact on chloroplast function since *in vitro* studies have shown that betaine can increase the thermal stability of photosystem II (PSII; (5)) and can protect against the inhibitory effect of NaCl (9). Krall *et al* (16) have shown that betaine can stabilize the active tetrameric form of phosphoenolpyruvate carboxylase which normally forms inactive dimers when exposed to low temperature. Betaine accumulation in the chloroplasts may be an important factor that could play a significant role in maintaining chloroplast function at low temperature. It is worth noting that hardy cereals such as rye, wheat, and barley have higher basal levels of betaine compared to sensitive species such as rice, millet, and sorghum (11).

To determine whether exogenous betaine could play a role in improving FT and photosynthesis at low temperature, we first evaluated the plant's capacity to accumulate betaine. In the first experiment, we incubated plants in 500 mM betaine and determined the osmolality of the leaves at different periods. The osmolality was found to increase rapidly during the first two days and levelled off thereafter (result not shown). We repeated the experiment using different concentrations of betaine and quantified the amount of betaine accumulated in the leaves after a four day period. The method described in (17) was used to extract betaine from 1 g of leaf tissue. Quantitation was performed according to Lerma *et al* (18). For accurate evaluation, an internal standard was added before the extraction procedure. The betaine content was expressed in mOsm/kg H₂O considering the tissue water content for each sample (an average of 82% water content was obtained). Osmolality was measured from leaf tissue after grinding with a mortar and pestle. The liquid obtained was centrifuged at 12,000 g for 10 min at 4°C. The osmolality was evaluated in the supernatant using a Wide Range Osmometer. We found that betaine accumulated efficiently at all concentrations used. The accumulated betaine (expressed in mOsm/Kg H₂O) was equivalent to 62% of the external betaine when exposed to betaine concentrations ranging from 118 to 590 mM (100 to 500 mM). Betaine could accumulate even more at higher concentrations, however, signs of chlorosis at the leaf tips became evident at 500 mM. Chlorosis became even more extensive when higher betaine concentrations were used.

Betaine accumulation reduced the growth rate in a manner proportional to the amount of exogenous betaine applied. At the highest concentration used, the growth was reduced by 75% over the 4 day incubation period compared to control plants. The reduction in growth and more importantly, the increase in cellular betaine content was found to be associated with a substantial increase in survival rate after freezing compared to control non-acclimated plants (Fig. 1A). Interestingly, both cultivars are protected by betaine with only a slight advantage in the more tolerant cultivar at all concentrations used (not shown). Plant survival is increased even when a relatively low concentration of betaine is used. At 100mM, survival improved by 5-6 fold compared

to the untreated plants (Fig. 1A). Treating with 250 mM betaine alone was sufficient to increase the FT of the spring cultivar Glenlea from -3°C to -8°C. This value corresponds to the maximal FT achieved by this cultivar after 4 weeks of cold acclimation (Fig. 1B). Increasing the concentration of betaine to a higher concentration
5 resulted in a slightly higher survival rate (corresponding to 55% survival at 500 mM betaine; Fig. 1A) but due to the toxicity, of higher betaine concentrations, the latter were eliminated in other experiments. We have examined whether treatment with betaine during cold acclimation could improve FT in the less tolerant cultivar Glenlea submitted to cold-acclimating conditions. Figs. 1A and 1B show that the survival of
10 plants treated with betaine during cold acclimation were dramatically improved over plants that are cold acclimated in the absence of betaine. Fig. 2 shows the results of a typical experiment for plants treated with betaine at 25°C or during cold acclimation. Betaine treatment at 25°C for 4 days allowed the plants to reach an LT₅₀ of -8°C (the maximal LT₅₀ normally achieved by this cultivar) while those treated with betaine during
15 cold acclimation were barely affected by a temperature of -13°C (the average LT₅₀ was estimated as -14°C in Fig. 1B). These results demonstrate that the improvement in FT observed in control plants exposed to betaine is additive in cold-acclimated plants. This finding is of crucial importance since it is the first time that the normal genotypic potential to tolerate freezing has been improved so dramatically. We have also
20 evaluated the capacity of betaine to improve FT in barley which was also shown to accumulate betaine upon cold acclimation and found that the combined treatment of low temperature and betaine was as efficient in this species as in wheat to improve FT.

It is therefore expected that the improvement in cold or freezing tolerance will be observed in almost all plants, particularly gramineae or grasses, more particularly
25 cereals such as rice, corn, rye, wheat, barley and oat. The conditions at which such improvement will occur are set as follows. The plants are acclimated at the coldest temperature that they can withstand. Betaine is administered before, during and at the end of the cold acclimation. The dosage regimen of betaine is determined on test plants in order to evaluate the doses at which betaine is toxic, in such a way that
30 unacceptable toxic doses will be further avoided. We expect that even tropical plants which are very cold-sensitive will benefit form a combined treatment.

Furthermore, since the growth rate of the plants were reduced down to 25% the control plants, it is readily apparent that, if betaine is applied at the end of cold acclimation (in field, that would mean during fall wherein the maximal growth is attained), this effect would not be deleterious to the plants. Moreover, this property may be advantageously used at the beginning or during the growing period, to slow down the growth of many plants. Thus, it could be used as a growth-retarding substance for several agronomical applications. A specific useful application would be found for golf courses to reduce the maintenance costs (it would reduce the number
35

of times one has to mow the grass during a season). At higher toxic or lethal doses, betaine could even be used as a herbicide to inhibit the growth of undesirable plants or kill them.

Treatments with other osmolytes such as NaCl or mannitol allowed the
5 osmolality to increase as much as with betaine treatment. However, the FT was not significantly improved when these osmolytes were used indicating that betaine specifically improves FT. These results suggest that the improvement in LT₅₀ of 6°C cannot be explained solely by the osmolyte role of betaine (at a concentration of 250 mM, betaine would depress the freezing point of water by 0.32°C). We thus
10 investigated whether betaine can induce a number of genes known to be associated with the development of FT. We examined the expression of three different proteins induced by low temperature using specific antibodies and immunoblot analysis as well as the expression of three other low temperature-induced genes using northern analysis. Our results showed that the protein WCOR410 (Genbank accession no.
15 L29152) accumulates in a concentration-dependent manner when plants are exposed to varying amounts of betaine (Figure 3). When we examined the expression of the WCS120 (Genbank accession no. M93342) protein family or of the WCS19 (Genbank accession no. L13437) protein, we found that these proteins did not accumulate upon exposure to betaine (not shown). Since these two genes are known to be specifically
20 induced by low temperature and that the gene WCOR410 was inducible by low temperature, salinity, and water stress (19), we examined the possibility that other genes induced by drought or salinity are also induced by the presence of betaine. The genes WCOR80 (Genbank accession no. U73212), WCOR413 (Genbank accession no. U73216), and WCOR825 (Genbank accession no. U73215) were only slightly
25 inducible by betaine (not shown). These results suggest that the WCOR410 gene is specifically induced to high levels by betaine exposure and may be a major contributor to the improvement of FT above the level that could be explained solely by the role of betaine as an osmolyte. The WCOR410 protein was found to be associated with the plasma membrane. This is the first abundant protein found in the membrane fraction
30 of the vascular tissues (20), a region of the seedling known to be the most freezing sensitive tissue in wheat (21).

Betaine was shown to protect thylakoid membranes from freezing stress *in vitro* (22). Furthermore, in glycine-betaine deficient maize lines, high temperature decreases membrane stability and the resistance to photoinhibition as well as the
35 steady-state yield of electron transport over PSII (23). These results suggest that betaine may confer greater membrane stability under both low and high temperature. It can also protect the photosystem II against salt stress *in vitro*. Since betaine is normally synthesized in the chloroplast, it may accumulate to a higher concentration

in this organelle as suggested (8, 14). Thus, we evaluated the effect of the exogenous supply of betaine on the resistance to photoinhibition and oxygen evolution.

During steady-state photosynthesis at the prevailing growth conditions, exposure of spring and winter wheat to 250 mM betaine resulted in small but 5 consistently higher levels of qP and higher yields of PSII electron transport (Φ_e) than non-treated controls (Table I). Thus, betaine treated spring and winter wheat seedlings appeared to exhibit a greater capacity to prevent the reduction of PSII reaction centres than non-treated controls. The increased capacity to keep PSII reaction centres oxidized was correlated with a decreased susceptibility to low temperature (5°C) 10 photoinhibition (measured according to 24) in betaine-treated seedlings. The Fv/Fm ratio of 250 mM betaine-treated plants submitted to high light (2h at 5°C and 1600 $\mu\text{mol m}^{-2}\cdot\text{s}^{-1}$) was of 84 ± 4% of non-photoinhibited control plants compared to 72 ± 2% for plants not treated with betaine. This effect of betaine on the photosynthetic machinery may improve the physiology and performance of the plants at low 15 temperature and thus provide the plants with a greater capacity to use the available energy to develop FT.

Betaine application early in the fall improves the performance of golf turf and consequently increased winter survival. The betaine-treated turf showed a rapid regrowth in the spring indicating a higher winter survival rate and healthier plants at 20 spring which have a better regrowth rate.

Based on the above results and based on the partial teachings of WO 95/35022, it is further expected that the germination rate of plant seeds can be improved at cold temperature. This method will comprise the steps of administering to the seeds an effective dosage regimen of betaine or derivative thereof, and allowing 25 the same to germinate at cold temperatures. Such cold temperatures would be above about 0°C but not lower than the coldest temperature that the one plant seeds can withstand.

An important conclusion one can draw from these observations is that it may be possible to increase FT by using only one or two of the major genes (such as 30 Wcor410) associated with this multigenic trait. Thus, it may be possible to increase the FT of cold sensitive species by transforming plants with this gene. Furthermore, our results suggest that FT could be improved in an additive manner in plants that already possess some degree of FT by overexpressing the most important genes involved in the process of FT. For example, overexpressing betaine dehydrogenase and choline 35 monooxygenase under a low temperature promoter may allow the accumulation of betaine at the time where it could help against freezing stress. Such manipulations of betaine production could have a great agronomic and economic impact not only for protection against drought stress as previously suggested but also for FT as shown in this report. In addition, the results presented in this report indicate that exogenous

application of betaine before a predicted frost or sudden decrease in temperature may be exploited on a short-term basis to increase the resistance of cold sensitive plants to low temperature stress.

This invention has been described in details hereinabove, and it is readily apparent that modifications thereto can be made, without departing from the spirit of the invention and from the above teachings. These modifications fall under the scope of the invention as defined in the appended claims.

REFERENCES

1. D. Rhodes, A. D. Hanson. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **44**: 357-384 (1993).
2. A.D. Hanson, B. Rathinasabapathi, J. Rivoal, M. Burnet, M.O. Dollon, D.A. Gage. *Proc. Natl. Acad. Sci. (U.S.A.)* **91**: 306-310 (1994).
- 5 3. K. F. McCue, A. D. Hanson. *Trends Biotech.* **8**: 358-362 (1990).
4. P. H. Yancey, M. E. Clark, S. C. Hand, R. D. Bowlus, G. N. Somero. *Science* **217**: 1214-1222 (1982).
5. Y. Jolivet, F. Larher, J. Hamelin. *Plant Sci. Lett.* **25**: 193-201 (1982).
- 10 6. A. Shomer-Ilan, G. P. Jones, L. G. Paleg. *Aust. J. Plant Physiol.* **18**: 279-286 (1991).
7. K. B. Schwab, D. F. Gaff. *J. Plant Physiol.* **137**: 208-215 (1990).
8. H. Génard, J. Le Saos, J. -P. Billard, A. Trémolières, J. Boucaud, *Plant Physiol. Biochem.* **29**: 421-427 (1991).
- 15 9. N. Murata, P. S. Mohanty, H. Hayashi, G. C. Papageorgiou. *FEBS Lett.* **296**: 187-189 (1992).
10. C. Itai, L. G. Paleg. *Plant Sci. Lett.* **25**: 329-335 (1982).
11. S. Kishitani, K. Watanabe, S. Yasuda, K. Arakawa, T. Takabe. *Plant, Cell environment* **17**: 89-95 (1994)
- 20 12. R. G. Wyn Jones, R. Storcy, R. A. Leigh, N. Hamad, A. Pollard. In: *Regulation of Cell Membrane Activities in Higher Plants*, ed. E. Marrè, O. Ciferri, pp. 121-136. Amsterdam: Elsevier/North Holland (1977).
13. T. Matho, J. Watanabe, E. Takahashi. *Plant Physiol.* **84**: 173-177 (1987).
14. S. P. Robinson, G. P. Jones. *Aust. J. Plant Physiol.* **13**: 659-668 (1986).
- 25 15. W. P. Williams, A. P. R. Brian, P. J. Dominy. *Biochim. Biophys. Acta* **1099**: 137-144 (1992).
16. J. P. Krall, G. E. Edwards, C. S. Andreo. *Plant Physiol.* **89**: 280-285 (1993).
17. D. Rhodes, P. J. Rich, A. C. Myers, C. C. Reuter, G. C. Jamieson. *Plant Physiol.* **84**: 781788 (1987).
- 30 18. C. Lerma, P. J. Roch, G. C. Ju, W. J. Yang, A. D. Hanson, D. Rhodes. *Plant Physiol.* **95**: 1113-1119 (1991).
19. J. Danyluk, M. Houde, É. Rassart, F. Sarhan, *FEBS Lett.* **344**: 20-24 (1994).
20. J. Danyluk (1996) Identification et caractérisation moléculaire de gènes induits au cours de l'acclimatation au froid chez le blé (*Triticum aestivum*). Ph. D. thesis. Université de Montréal. Montréal, Québec, Canada.
- 35 21. K. K. Tanino, B. D. McKersie. *Can. J. Bot.* **63**: 432-436 (1984).
22. S.J. Coughlan, U. Heber, U. *Planta* **156**: 62-69 (1982).
23. G. Yang, D. Rhodes, R. J. Joly. The American Society of Plant Physiologists. San Antonio, Texas, U.S.A (1996).

24. A. G. Ivanov, M. Krol, D. Maxwell, N. P. A. Huner. *FEBS Lett.* **371**: 61-64 (1995).
25. M. Perras, F. Sarhan. *Plant Physiol.* **89**: 577-585 (1989).

**The embodiments of th invention in which an exclusive property or
privil ge is claimed are defined as follows:**

1. A method of increasing cold or freezing tolerance in a plant, which comprises the steps of:

5 - acclimating said plant to a temperature higher than about 0°C but not lower than the coldest temperature that said plant is capable to withstand, for a time sufficient to induce an optimal cold or freezing tolerance, in said plant, and

10 - administering a composition comprising betaine or a derivative thereof to said plant, in a dosage regimen sufficient to induce the same or different optimal cold or freezing tolerance in said plant;

whereby combined steps of cold-acclimating and administering betaine or derivative thereof increase cold or freezing tolerance of said plant over and above the optimal cold or freezing tolerance induced by each step alone.

15 2. A method as set forth in claim 1, wherein said dosage regimen is non-toxic to said plant.

3. A method as set forth in claim 1 or 2, wherein said plant is selected from gramineae and grasses.

4. A method as set forth in claim 3, wherein said gramineae is barley or wheat.

20 5. A method as set forth in claim 4, wherein said time for cold-acclimating is about four weeks.

6. A method as set forth in any one of claims 1 to 4, wherein betaine derivative is glycine betaine.

7. A method as set forth in claim 5, wherein betaine derivative is glycine betaine.

25 8. A method as set forth in claim 7, wherein said dosage regimen is growing said plant in the presence of a solution of glycine betaine having a concentration lower than about 500 mM.

9. A method as set forth in claim 8, wherein said concentration is about 250 mM.

10. A method as set forth in claim 9, wherein said plant is the spring wheat variety Glenlea, in which the optimal freezing tolerance, expressed as the temperature where fifty percent of a plant population die (LT_{50}) is about -8°C for each step alone.
- 5 11. A method as set forth in claim 10, wherein the increase of freezing tolerance is by about 6°C to reach a LT_{50} of about -14°C .
12. A method as set forth in claims 10 or 11, wherein the optimal freezing tolerance induced by said each step alone or in combination is due at least in part to an increased expression of the gene *Wcor410*.
- 10 13. A method as set forth in claim 9, which further results in improving photosynthetic capacity and overall physiology of said plant at cold temperature.
14. A method of reducing the growth rate of a plant by at least 30%, thus having a growth-retarding effect which comprises the step of treating said plant with an effective dosage regimen of a composition comprising betaine or derivative thereof which is not lethal to said plant.
- 15 15. A method as set forth in claim 14, wherein said dosage regimen of betaine or derivative thereof is not toxic to said plant.
16. A method according to claim 15, wherein said plant is golf course grass.
17. A method as set forth in claim 14, wherein said plant is spring wheat variety Glenlea.
- 20 18. A method as set forth in claim 17 wherein said dosage regimen is growing said plant in the presence of 500 mM of glycine betaine for four days which results in a growth rate reduction by about 75%.
- 25 19. A method of killing a plant, which comprises the step of treating said plant with a composition comprising betaine or derivative thereof, thus having a herbicidal effect thereon at a lethal dosage regimen.
20. A method according to claim 19 wherein said plant is a dicotyledon.
21. A method according to claim 20, wherein said plant is selected from the group consisting of dandelion, canola, alfalfa, strawberry and tobacco.

22. A method of stimulating and improving the germination rate of plant seeds at a temperature which is higher than about 0°C but not lower than the coldest temperature that said plant seeds can withstand, which comprises the steps of administering to said seeds a composition comprising betaine or derivative thereof at an effective dosage regimen, and allowing said seeds to germinate at said temperature.

5

23. A method of improving tolerance of a plant to an abiotic stress, which comprises the step of administering a composition comprising an effective amount of betaine or derivative thereof.

24. A method according to claim 23, wherein said stress is selected from the group
10 consisting of drought, salinity, cold and freezing temperatures.

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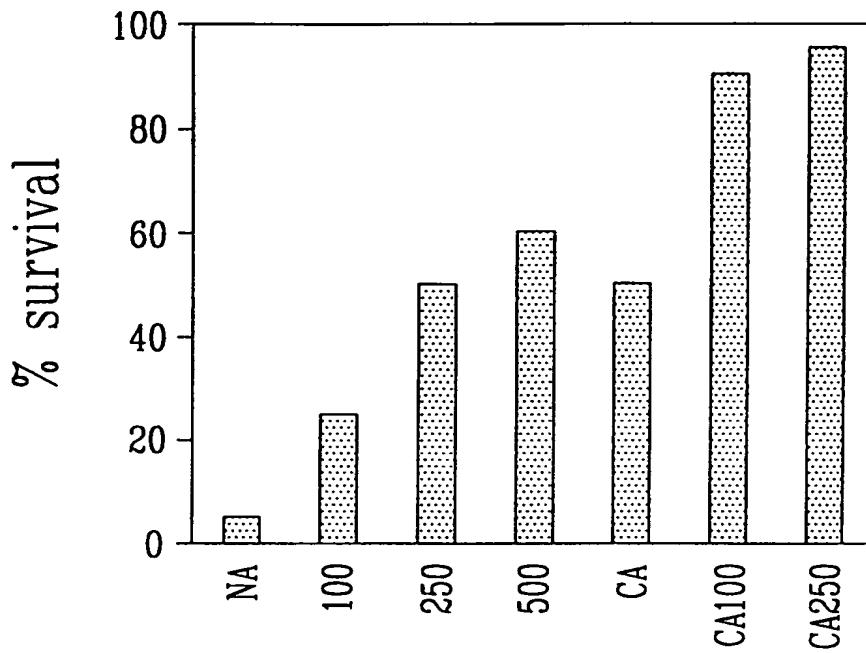


FIGURE 1A

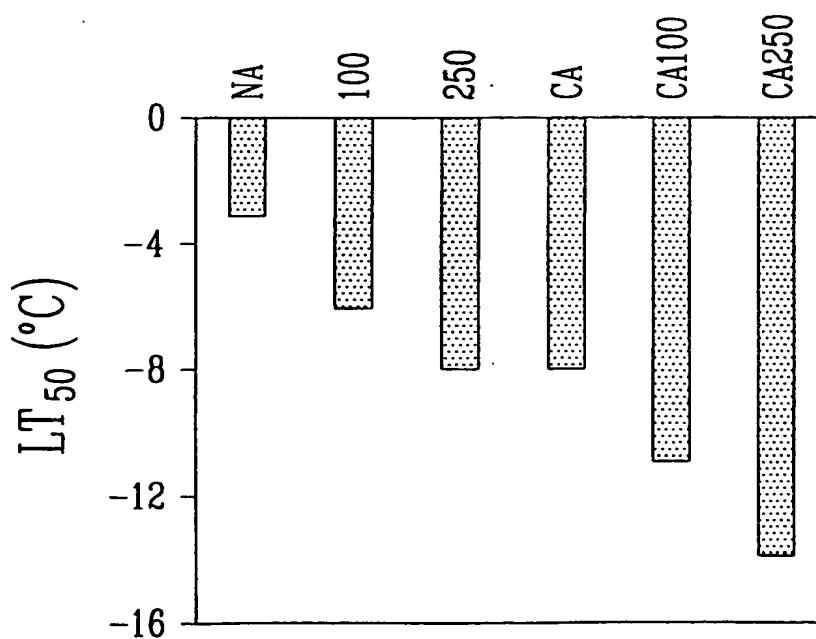


FIGURE 1B

215

A

N A

250



C A

C A 250

**B**

C A

C A 250



FIGURE 2

SUBSTITUTE SHEET (RULE 26)

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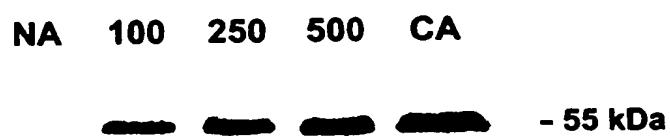


FIGURE 3

Figure 4 A

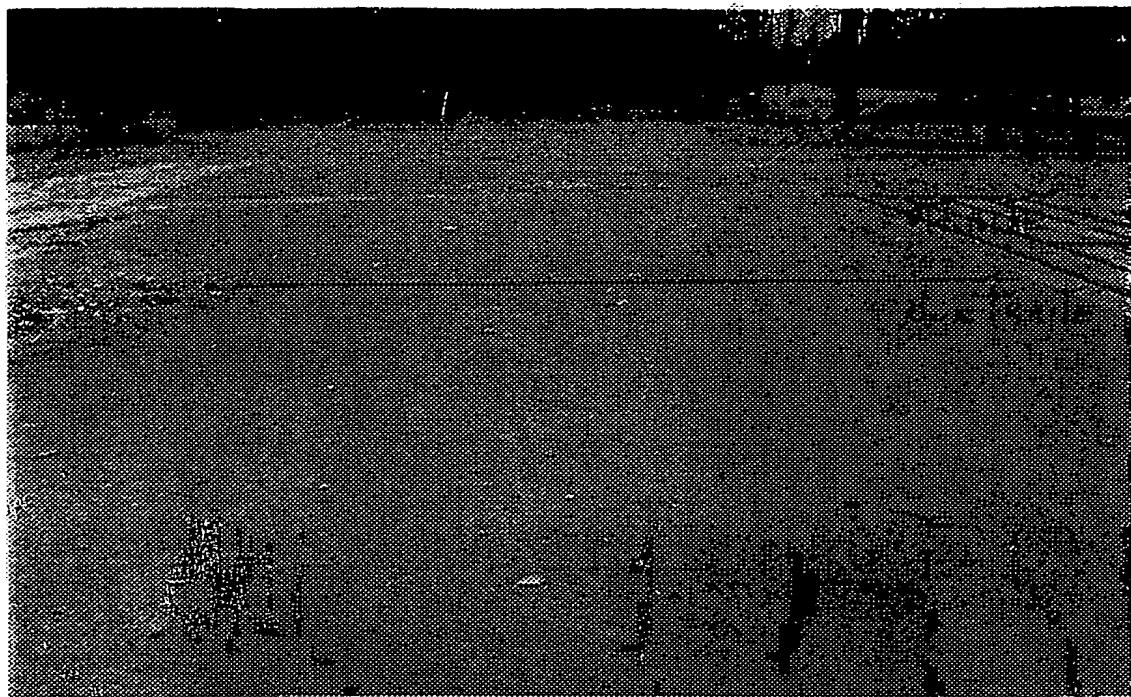


Figure 4 B

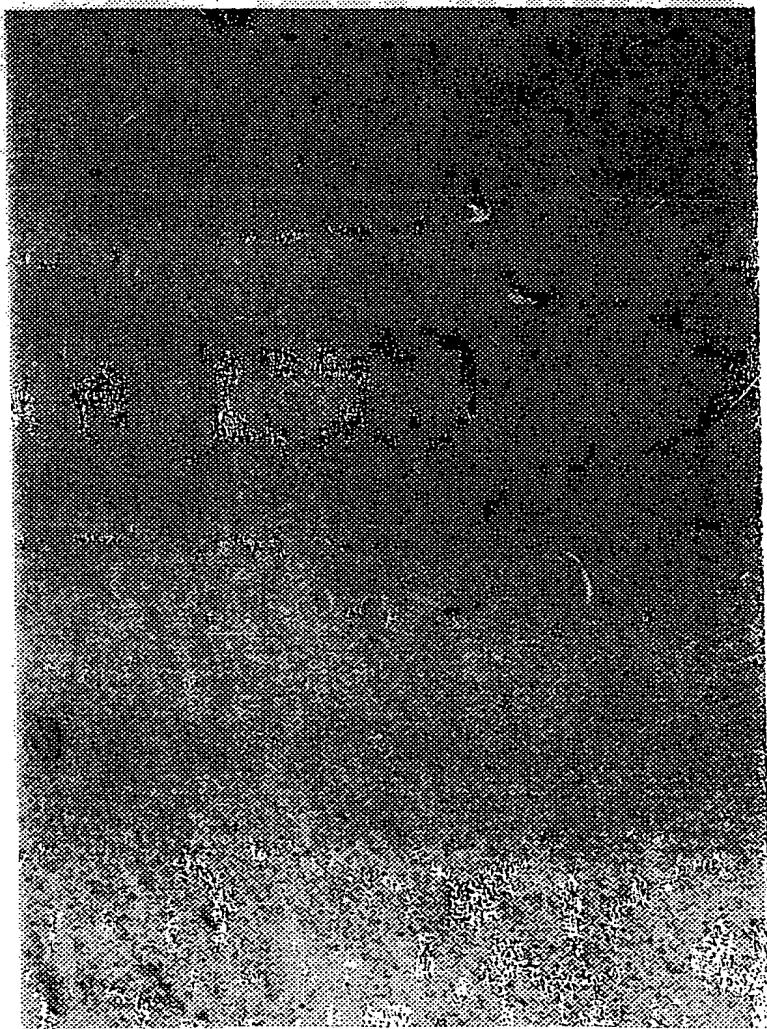


Figure 5 A

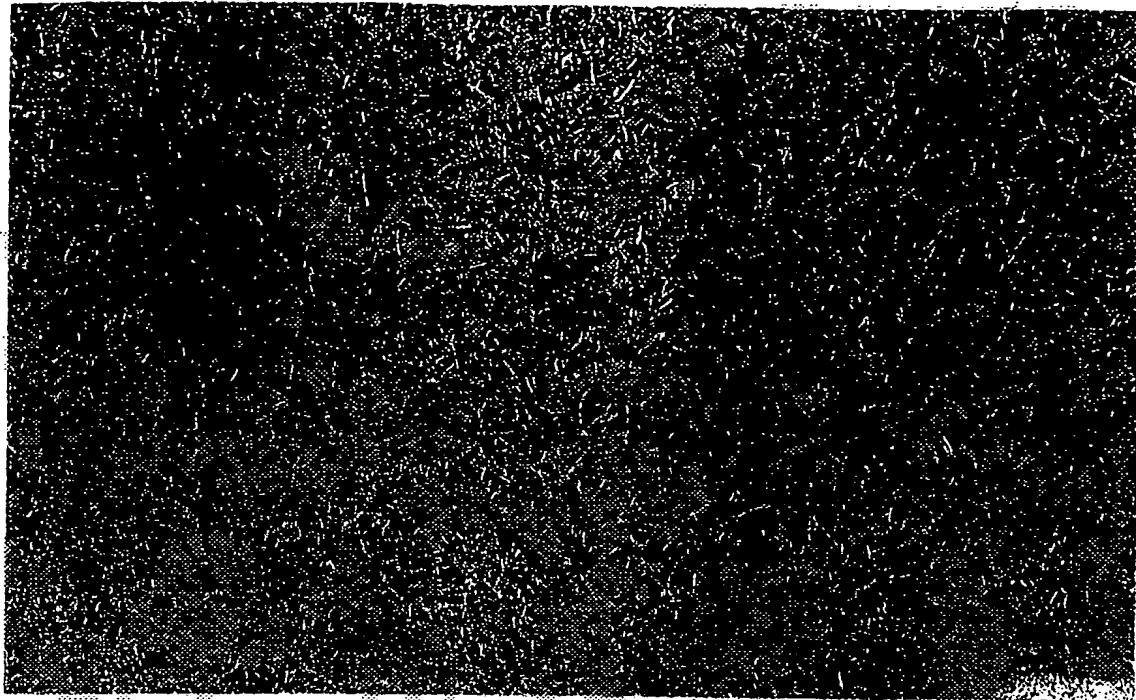
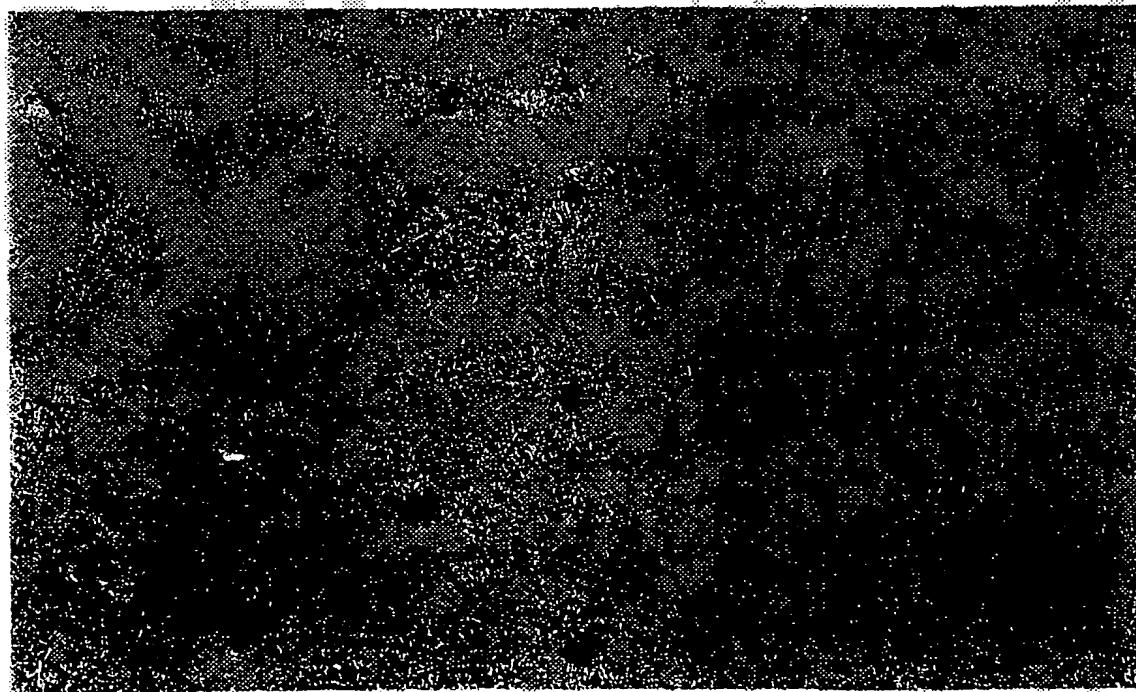


Figure 5 B



INTERNATIONAL SEARCH REPORT

In. national Application No

PCT/CA 98/00648

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 A01N37/44

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>CHEMICAL ABSTRACTS, vol. 120, no. 19, 9 May 1994 Columbus, Ohio, US; abstract no. 240489, KISHITANI, S. ET AL: "Accumulation of glycinebetaine during cold acclimation and freezing tolerance in leaves of winter and spring barley plants" XP002084394 see abstract & PLANT, CELL ENVIRON. (1994), 17(1), 89-95 CODEN: PLCEDV; ISSN: 0140-7791, 1994, ---</p> <p style="text-align: center;">-/--</p>	1-13

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

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Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/CA 98/00648

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 96 41530 A (CULTOR OY ;PEHU EIJA (FI); HAUTALA JUSSI (FI); KOKKONEN ESKO (FI)) 27 December 1996 cited in the application see page 1 - page 10, line 3 see claims 1-4; examples 5,6 ---	1-13
X		23,24
A	PERRAS MICHEL ET AL.: "Synthesis of Freezing Tolerance Proteins in Leaves, Crown, and Roots during acclimation of Wheat" PLANTPHYSIOLOGY, vol. 89, 1989, pages 577-85, XP002084392 cited in the application see page 577, the abstract ---	1-13
A	CA 2 104 142 A (LALIBERTE JEAN FRANCOIS ;HOUDE MARIO (CA); SARHAN FATHEY (CA)) 17 February 1995 cited in the application see page 1 - page 4, line 13 see page 30, line 14 - page 40, line 19 ---	1-13
A	KOSTER, KAREN L. ET AL: "Solute accumulation and compartmentation during the cold acclimation of Puma rye" PLANT PHYSIOLOGY, vol. 98, no. 1, 1992, pages 108-13, XP002084393 see page 108, the abstract see page 108, paragraph 1 - paragraph 3 ---	1-13
X	DATABASE WPI Week 7349 Derwent Publications Ltd., London, GB; AN 73-75195U XP002084395 & SU 369 887 A (KA TIMIRYAZEV PLANT PHYS.) see abstract ---	14-18
X	EP 0 017 946 A (BASF AG) 29 October 1980 see page 1, line 4 - line 9 see page 4 - page 7; examples 5-7 ---	14,15, 23,24
X	US 4 032 325 A (KIDA TAKAO ET AL) 28 June 1977 see column 1, line 29 - column 2, line 61; example 3; table 5 ---	19-21
X	WO 96 41532 A (CULTOR OY ;VIRTANEN ERKKI (FI); PEHU EIJA (FI)) 27 December 1996 see page 1 - page 10, line 21; claims; examples 2,4,5 ---	22-24
	-/-	

INTERNATIONAL SEARCH REPORT

In	ational Application No
PCT/CA 98/00648	

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 08951 A (CULTOR OY ; VIRTANEN ERKKI (FI); PEHU EIJA (FI)) 13 March 1997 see examples 2,4,5 ---	22-24
X	WO 95 35022 A (COMMW SCIENT IND RES ORG ; BODAPATI PURUSHOTHAMA NAIDU (AU)) 28 December 1995 cited in the application see page 1 - page 13, line 27; claims -----	22-24

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/CA 98/00648

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO 9641530 A	27-12-1996	FI 952865 A AU 3348195 A CA 2223556 A CN 1191467 A CZ 9703967 A EP 0831699 A PL 323820 A		10-12-1996 09-01-1997 27-12-1996 26-08-1998 13-05-1998 01-04-1998 27-04-1998
CA 2104142 A	17-02-1995	US 5731419 A		24-03-1998
EP 0017946 A	29-10-1980	DE 2915250 A AT 850 T BR 8002273 A CA 1140122 A JP 55160754 A US 4360465 A		30-10-1980 15-04-1982 02-12-1980 25-01-1983 13-12-1980 23-11-1982
US 4032325 A	28-06-1977	JP 50111235 A JP 922910 C JP 50069225 A JP 53001816 B GB 1455963 A US 4102672 A		01-09-1975 22-09-1978 10-06-1975 23-01-1978 17-11-1976 25-07-1978
WO 9641532 A	27-12-1996	FI 952866 A AU 3348395 A CN 1191466 A EP 0831701 A		10-12-1996 09-01-1997 26-08-1998 01-04-1998
WO 9708951 A	13-03-1997	FI 98515 B AU 3348095 A EP 0863703 A		27-03-1997 27-03-1997 16-09-1998
WO 9535022 A	28-12-1995	AU 683177 B AU 2707195 A		30-10-1997 15-01-1996

